Neth. J. Pl. Path. 91 (1985) 215-223

Factors governing oospore production by Peronospora farinosa f. sp. spinaciae in cotyledons of spinach

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Accepted 12 June 1985

Abstract

Oospore production in *Peronospora farinosa* f. sp. *spinaciae* was induced in spinach seedlings. This was done in detached cotyledons as wel as in undetached cotyledons on living plants, and in growth chambers as well as in the field. In young leaf tissue the fungus produced oospores more often than in old tissue. The four spinach cultivars tested, viz. Breedblad Scherpzaad, Symphony, Novires and Huro, did not show significant differences in numbers of cotyledons with oospores, nor in numbers of oospores per cotyledon, though cv. Breedblad Scherpzaad was less susceptible to oospore formation in the field. Comparison of various inoculum densities showed a tendency to a smaller number of cotyledons with oospores with a lower inoculum density. Oospores were abundantly formed, when the plants were exposed to stress conditions in the second half of the latent period.

Additional keywords: downy mildew.

Introduction

Spinach is a crop with high commercial value and is cultivated for direct consumption and industrial processing. Growers are increasingly complaining about the lack of information on primary infection by downy mildew resulting in disastrous epidemics and important losses.

Several means of overwintering by the downy mildew fungi have been proposed and discussed in the past. Populer (1981) summarized the various publications on this subject. The possibilities for these fungi to overwinter strongly depend on host range, cultural practices and climatic conditions. In principle the life cycle of all downy mildews can include a sexual stage. The oospores can survive during adverse climatic conditions.

For *Peronospora farinosa* (Fr.) Fr. f. sp. *spinaciae* [Byford], the causal agent of downy mildew on spinach *(Spinacia oleracea* L.), the role of oospores in hibernation of the pathogen is greatly underestimated. The reason is twofold. Firstly, the assortment of spinach varieties has been expanded, so that spinach can be grown in the open nearly the whole year round in temperate climates. In combination with a long latent and infectious period of the fungus of sometimes over 20 to 40 days at 0 to 5 °C (Frinking, unpublished), this gives rise to the hypothesis that the onset of a new epidemic in early spring fits in a continuous series of asexual reproduction cycles. If so, the an-

nual infection cycle of the fungus, in fact being of the nested-type, shifts to the serial-type infection cycle (Zadoks and Schein, 1979). Secondly, there is a lack of knowledge about the conditions under which formation of and infection by oospores take place.

Oospores of *P. farinosa* f. sp. *spinaciae* have been found frequently. Cook (1935) already mentioned the presence of oospores in commercial spinach seed. His paper, however, does not give any certainty about the exact place where the oospores have been found: in the seeds or on their surface. Internal seed transmission was not demonstrated beyond doubt.

Wright and Yerkes (1950) indicated the importance of oospore contaminated soils as a source of primary infection. Infected leaves can be ploughed in, which is common when a spinach crop is attacked seriously and considered to be lost.

In the literature several observations and studies have been published on the conditions favouring the formation of oospores in different *Peronospora* species. These statements partly or completely fit together and can be compiled as follows:
(1) oospores are formed under conditions favouring senescence of host tissue (McMeekin, 1960; Melhus and Patel, 1929).
(2) oospores are abundantly formed in chlorotic and necrotic host tissue (McMeekin, 1960; Pawlik, 1961).
(3) oospores are frequently found in cotyledons which fade rapidly (Channon, 1981).
(4) oospores are formed under climatic conditions unfavourable for asexual reproduction (Berry and Davis, 1957; Pegg and Mence, 1970; Populer, 1981).
(5) oospores are easily formed in detached plant material under humid conditions (Inaba and Hino, 1980; Norwood and Crute, 1983).

These observations on different *Peronospora* species lead to the conclusion that oospore production can be induced by factors causing stress at the moment that fungal colonization inside the leaf has been completed. There is no reason to assume that *P. farinosa* f. sp. *spinaciae* would respond differently.

The present study describes the conditions conducive to oospore formation. Their significance as a source of primary infection is considered.

Materials and methods

Techniques to inititate oospore formation in spinach seedlings were based upon those of Norwood and Crute (1983), for *Bremia lactucae* on lettuce. The techniques include one for the formation in detached plant material, especially cotyledons, one for the formation in undetached plant material in growth chambers and one for the formation in undetached plant material under field conditions.

Detached plant material. Cotyledons were detached from spinach seedlings (one cotyledon per seedling chosen at random). The cotyledons were placed in petri dishes on two layers of wet filter paper. The amount of water in the dishes was not crucial as it was meant to ensure a high relative air humidity in the dish. An experiment with different quantities of water in the petri dishes (from 1 to 3 ml) did not show significant differences in numbers of oospore-containing cotyledons. The cotyledons were inoculated with a conidiospore suspension of *P. farinosa* f. sp. *spinaciae* by means of a DeVilbiss atomizer until these were covered with a thin layer of the inoculum. After inocultion, the petri dishes were closed and kept in growth chambers at 15 °C for 10

days, with a 16 h day (at a light intensity of 7 to 8 klx) and an 8 h night. During incubation the cotyledons slowly yellowed.

Undetached plant material (growth chamber experiments). Seedlings were grown in plastic pots (ø 12 cm). During emergence the number of seedlings was reduced to 10 seedlings of about the same development stage per pot. The cotyledons of these seedlings were inoculated with a conidiospore suspension as described above. After inoculation the pots were placed in plastic nursery trays (46×31×15 cm). The trays were covered for 24 h to stimulate germination and penetration. Thereafter the trays were uncovered and the plants were kept under conditions of water stress. The pots were hardly watered and the soil was left to dry out slowly. The experiments were performed in growth chambers at 15 °C, a relative humidity of 70 to 80%, a 16 h day (at about 7 klx at cotyledon level) and an 8 h night. After an incubation period of 10 days the observations could take place.

Undetached plant material (field experiments). Seedlings were grown as described before, but in this case Jiffy pots were used. After sowing, the pots were transported to the field and dug in. Inoculation was scheduled at sunset so that a night of low temperatures (± 5 °C) and high relative air humidities (>80%) could follow to stimulate germination and penetration. Observations on oospore formation were made 14 days after inoculation.

Inoculum. For all experiments an inoculum was used consisting of a mixture of conidia from different isolates of pathotype 3 of *P. farinosa* f. sp. *spinaciae.* The conidiospore suspensions were made by shaking sporulating leaves in demineralized water. The suspension was filtered through cheese-cloth. The inoculum densities were different for growth chamber experiments (3.10^5 conidia ml^{-1} water and 10- and 100-fold dilutions) and for field experiments (17.10^6 conidia ml^{-1} water and 10- and 100-fold dilution). The reason for using a higher inoculum density in the field was a previously experienced reduction of germinated conidia and penetrated germ tubes under field conditions. Besides, plant leaves in the field are shaken by air movements. This causes a confluence of the inoculum droplets, a flow of inoculum to the leaf axil and consequently a loss of inoculum.

Plant material. Four cultivars of spinach were used, viz. cvs Breedblad Scherpzaad (from Rijk Zwaan B.V., De Lier), Symphony (from Royal Sluis, B.V., Enkhuizen), Novires (from Van der Ploeg Elite Zaden B.V., Barendrecht) and Huro (from Nunhems Zaden B.V., Haelen). All four cultivars are susceptible to pathotype 3 of *P. farinosa* f. sp. *spinaciae,* but only cv. Breedblad Scherpzaad is susceptible to pathotypes 1 and 2 as well. They are recommended to be grown in different periods of the year in the Netherlands.

Observation method. On the day of observation the cotyledons were placed in petri dishes on a double layer of filter paper wetted with a 3 : 1 (v/v) mixture of ethanol (96%) and glacial acetic acid (c. 100%) to decolourize the plant tissue within 24 h at room temperature. The yellow to orange oospores could easily be observed at a magnification of 50 to 100x.

For counting the oospores the cotyledons were pulverized in water, using an Ultra Turrax grinder, after which the plant tissue with the oospores was washed through sieves of subsequently 1000, 100 and 22 μm pores. Particles left on the last sieve were centrifuged at a RCF of 700 *g* for 5 min. after which the loose pellet was suspended in 5 ml tap water. Oospores were counted of 4 droplets of 25 μl each.

Statistical analysis. Experiments were carried out in triplicate with the following factors: inoculum density, seedling age and cultivar. These factors were tested simultaneously in experiments on detached spinach cotyledons in petri dishes (Exp. 1) and on detached cotyledons in a growth chamber (Exp. 2), both under controlled environmental conditions. A field experiment with undetached cotyledons (Exp. 3) was performed in June 1984. The results were recorded as:

OCC = number of cotyledons containing oospores (Exps 1 to 3)
NO/OCC = number of oospores per oospore-containing cotyledon (Exp. 1)
NO/C = number of oospores per cotyledon examined (Exp. 1)

The smallest statistical unit was 10 cotyledons. The data were subjected to an analysis of variance (ANOVA) after an $\arcsin\sqrt{p}$ (for OCC) or $\sqrt{p}$ (for NO/OCC and NO/C) transformation. This was necessary to consider the data as being random samples of a normal distribution. Main factors as well as interactions between pairs and triplets of factors were subject of the analysis. Significance of treatment differences was established to Tukey's comparison method (Snedecor and Cochram, 1980).

Results

Weather conditions during the field experiment. Environmental factors in the open can be rather variable but during Exp. 3 weather conditions were stable. The key-factors for asexual reproduction, mean air-humidity and rainfall, were too low (80% RH) and nearly absent (only 2 mm of rain at midday during 1.6 h), respectively. Sunshine was frequent and increased to 10 to 12 h per day during the last days of the incubation period, and temperatures reached daily peaks of over 25 °C. This situation caused a high stress for the small plants, which failed to grow and showed severe wilting of the cotyledons. Such conditions are conducive to oospore formation.

Inoculum density. In all three experiments (Tables 1 and 2), a significant difference was found for different inoculum densities between the numbers of oospore-containing cotyledons (OCC) and in Exp. 1 also between the number of oospores per oospore-containing cotyledon (NO/OCC).

For undetached cotyledons there was a tendency to a smaller OCC with a lower inoculum density. For detached cotyledons, the medium inoculum density gave the highest OCC. An explanation for this phenomenon can be the interaction between the experimental factors 'inoculum density' and 'seedling age' (Table 3).

Seedling age. Seedlings were used 3, 4 and 5 wk after sowing. In Exp. 1, in which cotyledons were detached before inoculation, the leaf area was fixed at the moment of detachment. Physiological ageing of plant material, however, accelerates at the moment of detachment. Maximal leaf area seemed to be reached 3 wk after sowing as

Table 1. Numbers and percentages of cotyledons containing oospores and total numbers of cotyledons examined.

Experimental factor	Experiment 1 Detached cotyledons (petri dishes)					Experiment 2 Undetached cotyledons (growth chamber)					Experiment 3 Undetached cotyledons (field)				
	C	OCC	% OCC	5%	1%	C	OCC	% OCC	5%	1%	C	OCC	% OCC	5%	1%
Inoculum density	1080					1080					1080				
3.10^5 spores ml^{-1}	360	104	28.9	b	b	360	194	53.8	a	a					
3.10^4 spores ml^{-1}	360	152	42.2	a	a	360	104	28.9	b	ab					
3.10^3 spores ml^{-1}	360	30	8.3	c	c	360	26	7.2	c	b					
17.10^6 spores ml^{-1}											360	84	23.3	a	a
17.10^5 spores ml^{-1}											360	27	7.5	b	b
17.10^4 spores ml^{-1}											360	7	1.9	c	c
Seedling age	1080					1080					1080				
5 wk	360	41	11.2	c	c	360	104	28.9	ab	a	360	8	2.2	b	b
4 wk	360	97	26.9	b	b	360	128	35.6	a	a	360	47	13.1	a	a
3 wk	360	148	41.1	a	a	360	92	25.6	b	a	360	63	17.5	a	a
Cultivars	1080					1080					1080				
cv. Novires	270	57	21.1	b	a	270	90	33.3	ab	ab	270	38	14.1	a	a
cv. Symphony	270	57	21.1	b	a	270	61	22.6	b	b	270	40	14.8	a	a
cv. Breedblad Scherpzaad	270	79	29.3	a	a	270	70	25.9	ab	ab	270	6	2.2	b	b
cv. Huro	270	93	34.4	a	a	270	103	38.1	a	a	270	34	12.6	a	a

C = number of cotyledons examined; OCC = number of oospore-containing cotyledons; % OCC = percentage of oospore-containing cotyledons.
5% = significant difference at 5% level according to Tukey's test; 1% = significant difference at 1% level according to Tukey's test.

Tabel 2. Average number of oospores per cotyledon examined and per oospore-containing cotyledon for an experiment with detached cotyledons in petri dishes.

Experimental factor	Experiment 1 Detached cotyledons in petri dishes							
	C	OCC	NO/C ($\times 10^3$)	5%	1%	NO/OCC ($\times 10^3$)	5%	1%
Inoculum density	1080	286						
3.10^5 spores ml^{-1}	360	104	0.63	b	b	2.16	b	b
3.10^4 spores ml^{-1}	360	152	1.80	a	a	4.26	a	a
3.10^3 spores ml^{-1}	360	30	0.33	b	b	4.01	a	a
Seedling age	1080	286						
5 wk	360	41	0.18	c	c	1.58	c	c
4 wk	360	97	0.70	b	b	2.60	b	b
3 wk	360	148	1.88	a	a	4.57	a	a
Cultivars	1080	286						
cv. Novires	270	57	0.59	b	b	2.79	b	b
cv. Symphony	270	57	0.91	ab	b	4.32	a	a
cv. Breedblad Scherpzaad	270	79	0.77	b	b	2.62	b	b
cv. Huro	270	93	1.41	a	a	4.09	a	a

C = number of cotyledons examined; OCC = number of oospore-containing cotyledons; NO/C = number of oospores per cotyledon examined (average); NO/OCC = number of oospores per oospore-containing cotyledon (average).
5% = significant difference at 5% level, according to Tukey's test; 1% = significant difference at 1% level, according to Tukey's test.

observations showed.

In Exp. 1 cotyledons of 3 wk old produced more oospores and had a higher NO/OCC thans those of 4 and 5 wk old. Exp. 3 with undetached cotyledons in a field situation supported the results of Exp. 1, though 3 and 4 wk old cotyledons did not differ significantly. In Exp. 2 with cotyledons in a growth chamber situation, differences were not indicative of a special optimal seedling age. The percentage of cotyledons containing oospores (Table 1, Exp. 2) was relatively high in all three cases, compared with those of Exps 1 and 3.

Cultivars. The cultivars tested only seldom showed clear, significant differences, though in the field experiment (Exp. 3) cv. Breedblad Scherpzaad had a significantly smaller OCC than the other three cultivars. As far as the NO/OCC in Exp. 1 is concerned (Table 2) the result of cv. Breedblad Scherpzaad equalled that of cv. Novires. Both cultivars showed a NO/OCC which was about two third of the other two cultivars.

Discussion

The experiments demonstrate that oospore formation by *P. farinosa* f. sp. *spinaciae*

Table 3. Statistical data on the numbers of oospore-containing cotyledons, the numbers of oospores per cotyledon examined and the numbers of oospores per oospore-containing cotyledons.

Nature of effects	Df	Variance estimate				
		Exp.1			Exp. 2	Exp. 3
		OCC	NO/C	NO/OCC	OCC	OCC
Main factors						
Inoculum density (I)	2	38.16[1]	12.70[1]	8.05[1]	56.15[1]	21.30[1]
Seedling age (S)	2	29.09[1]	15.47[1]	11.87[1]	3.28[2]	31.99[1]
Cultivars (C)	3	3.56[2]	1.77	0.90	4.30[1]	7.92[1]
Bi-factorial interactions						
I × S	4	3.68[1]	1.35	0.44	1.26	3.76[1]
I × C	6	1.36	0.39	0.25	1.97	2.53[2]
S × C	6	1.07	0.61	0.47	2.09	6.95[1]
Tri-factorial interactions						
I × S × C	12	1.18	0.64	0.63	1.27	2.35[2]

Df = degrees of freedom; OCC = number of oospore-containing cotyledons; NO/C = number of oospores per cotyledon examined; NO/OCC = number of oospores per oospore-containing cotyledon.
[1] Significant at 1% level.
[2] Significant at 5% level.

cannot be considered a rare phenomenon. The fungus is capable to produce oospores when suitable environmental conditions and the right inoculum are available.

Butler and Jones (1961) quoted Ferraris, an Italian author, who in 1928 published an unrecovered paper in which he signalized the existence of oospores and in which he recommended to use crop rotation as a means to control the disease, and to lift and burn all infected plants. Wright and Yerkes (1950) reported that oospores are the main form of overwintering in Washington State (USA). The above does not imply that oospores will be produced under all circumstances. The method used in the experiments to induce oospore formation was successful. When enough free water or water vapour is available, asexual reproduction will take place as long as possible until all food reserves of the leaves have been used. This was demonstrated by a field experiment comparable to the one described, but carried out in September and October 1984 during a period with high precipitation (45 mm during the incubation period) and air humidity (89% RH; T_{day} = 14.5 °C/T_{night} = 8.5 °C). Only conidiospores could be found.

The experiments give the impression, that more oospores were produced in young plant tissue. In the experiment, where detached cotyledons were studied, age differences of 1 wk could give differences at a 1% confidence level in oospore production and in numbers of cotyledons with oospores (Exp. 1). It became less clear in situations, where plant tissue could develop during the incubation period, which was 10 to 14 days

(Exps 2 and 3). Seedling age then, becomes a vague conception. This can explain the relatively small differences in OCC in Exps 2 and 3, except for the 5 wk old seedlings in the field experiment of which 2.2% of the cotyledons examined showed oospores in rather low numbers.

In Exp. 2 the percentages of oospore-containing cotyledons were relatively high at all three seedling ages, compared with those in Exps 1 and 3. The reason might be the conditions conducive to an excellent germination and penetration at the time of inoculation. The age of the cotyledons does not seem to affect the production of oospores when the seedling is in optimal condition during infection. In general, however, there are good reasons to assume, that the hypothesis 'the older the tissue, the less (frequent) oospores' is applicable.

The experiments were performed with cotyledons. These have a special nutritive function on the plant. This does not imply that only cotyledons are suitable to oospore production by the fungus. Occasionally true leaves of spinach plants of Exp. 2 were inoculated with conidia and incubated in the same way as described. Oospores were abundantly found in leaves of different ages up to the third pair of true leaves.

The environmental conditions under which the growth chamber experiments were carried out can be compared with those in the open during June and July, as long as temperatures and daylength are concerned. The cultivars suitable for this period are Novires and to some extent Symphony. These two cultivars are typically slow growers, the other two on the contrary are fast-growing cultivars. Whether the relation between growing conditions and plant development already plays a dominating role in the seedling stage of the spinach plant is unknown. At any rate, at the moment of inoculation approximately equal cotyledon areas for all four cultivars and all three seedling ages could be measured. After 3 wk the cotyledons seemed to reach their maximum area and the first leaves appeared. The cultivars tested usually did not show clear significant differences, though cv. Breedblad Scherpzaad could be indicated as a cultivar which is less inclined to oospore production than the others.

Nothing is known about hetero- or homothallism of downy mildew of spinach. Fincham et al. (1979) claimed, that most species of the *Peronosporales* are heterothallic. Nevertheless reports are known about homothallic isolates of various *Peronospora* species (De Bruyn, 1937; Michelmore and Ingram, 1980). For the present experiments a mixture of different isolates of pathotype 3 was used, of which at least three were collected in separated areas of The Netherlands. this was to be sure that in any case, including sexual incompatibility, oospores could be produced. Further investigations to elucidate this matter are intended.

Acknowledgement

The authors are indebted to miss Karin Kramers for her assistance with the mathematical analysis of Exp. 1.

Samenvatting

Factoren, welke van invloed zijn op de oosporevorming door Peronospora farinosa f.sp. spinaciae in cotylen van spinazie

Methoden werden ontwikkeld om oosporenvorming door *Peronospora farinosa* f. sp.

spinaciae in kiemplanten van spinazie te induceren. Dit gebeurde met afgeplukte kiembladeren en in kiembladeren aan de levende plant, zowel in klimaatcellen als te velde.

In jonge kiembladeren werden vaker en meer oosporen gevormd, dan in oudere kiembladeren. De vier getoetste cultivars Breedblad Scherpzaad, Symphony, Novires en Huro vertoonden geen opvallende verschillen in aantallen kiembladeren met oosporen en aantallen oosporen per kiemblad, hoewel cultivar Breedblad Scherpzaad in het veld als minst vatbaar voor oosporenvorming naar voren kwam.

Vergelijking van verschillende inoculum dichtheden toonde een tendens aan voor een kleiner aantal cotylen met oosporen bij lagere inoculumdichtheden. Er werden overvloedig oosporen gevormd wanneer de planten in de tweede helft van de latentieperiode in een 'stress' situatie werden gebracht.

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